Atty. Docket No.: 4231/2055B **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Choong-Chin Liew	Examiner:	Juliet Switzer
Serial No.:	10/802,875		
Filed:	March 12, 2004	Group Art Unit:	1634
Titled:	Method for the detection of coronary artery disease related gene transcripts in blood		
		Conf. No.:	8234

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF Hongwei Zhang UNDER 37 C.F.R. §1.132

Sir:

I, **Hongwei Zhang**, Ph.D., hereby declare that:

1. I received a Ph.D. degree from the Institute of Medical Science at the University of Toronto in 2002, and a Master of Science degree from the Department of Immunology at the University of Toronto in 1995. In addition I received my Medical Degree from the University of Medical Sciences in Changchun China in 1989 and practiced as a staff physician for 4 years in Beijing prior to commencing my post graduate studies. I currently hold the position of Director of Biomarker Development at GeneNews Corporation (formerly ChondroGene Ltd., the Assignee of the application).

I am a trained molecular biologist experienced in developing methods to identify biomarkers which are indicative of a disease or condition, and in developing methods of using these biomarkers and products thereof as applied in the area of bladder cancer, amongst other conditions.

List of Publications:

K.W. Marshall, M.D., PhD., F.R.C.S., **H. Zhang, M.D., PhD.**, T.D. Yager Ph.D., N. Nossova M.D., Ph.D., A. Dempsey PhD., R. Zheng M.D., M. Han M.D. Ph.D., H.Tang M.Sc., S. Chao M.A.Sc, and C.C. Liew PhD. "Blood-based biomarkers for detecting mild osteoarthritis in the human knee" *OsteoArthritis and Cartilage (2005)* 861-871.

Zhang H, Marshall KW, Tang H, Hwang DM, Lee M, Liew CC. Profiling genes expressed in human fetal cartilage using 13,155 expressed sequence tags. Osteoarthritis Cartilage 2003;11:309-19.

Hongwei Zhang, C.C.Liew, K.Wayne Marshall. Microarray Analysis Reveals the Involvement of Beta-2 Microglobulin (B2M) in Human Osteoarthritis. Osteoarthritis and Cartilage 2002;10:950-60.

Doherty PJ, **Zhang H**, Manolopoulos V, Trogadis J, Tremblay L, Marshall KW. Adhesion of transplanted chondrocytes onto cartilage in vitro and in vivo. J Rheumatol 2000;27:1725-312.

Zhao YX, Lajoie G, **Zhang H,** Chiu B, Payne U, Inman RD. Tumor necrosis factor receptor p55-deficient mice respond to acute Yersinia enterocolitica infection with less apoptosis and more effective host resistance. Infect Immun 2000;68:1243-513.

Vaselios Manolopoulos, K. Wayne Marshall, **Hongwei Zhang**, Judy Trogadis, Louise Trembly and Paul J. Doherty. Factors affecting the efficacy of bovine chondrocyte transplantation in vitro. Osteoarthritis and Cartilage 1999;7:453-460.

Yi-Xue Zhao, **Hongwei Zhang**, Basil Chiu, Usulra Payne, Robert D. Inman. Tumor necrosis factor receptor P55 controls the severity of arthritis in experimental *Yersinia Enterocolitica* infection. Arthritis & Rheumatism 1999;42:1662-1672.

Paul J. Doherty, **Hongwei Zhang**, Louise Trembley, Vaselios Manolopoulos and K. Wayne Marshall. Resurfacing of articular cartilage explants with genetically-modified human chondrocytes *in vitro*. Osteoarthritis and Cartilage 1998;6:153-160.

Hongwei Zhang, Donna Phang, Ronald M. Laxer, Earl D. Silverman, Sueihua Pan, and Paul J. Doherty. Evolution of the T cell receptor beta repertoire from synovial fluid T cells of patient with juvenile onset rheumatoid arthritis. J. Rheumatol. 1997;24:1396-402.

Petro Lastres, Anihoa Letamendia, **Hongwei Zhang**, Carlos Rius, Nuria Almendro, UIIa RAab, Louis A. Lopez, Carmen Langa, Angels Fabra, Michelle Letarte and Carmelo Bernabeu. Endoglin modulates cellular responses to TGF-beta 1. J. Cell Biol. 1996;133:1109-1121.

Hongwei Zhang, Andrew R.E. Shaw, Allan Mak, and Michelle Letarte. Endoglin is a component of the Transforming Growth Factor (TGF)-beta receptor complex of human pre-B leukemic cells. J. Immunol. 1996,156:565-573.

2. I have read the non-final Office Action mailed May 16, 2007 in the above-referenced patent application.

In providing grounds for rejection of claims under 35 U.S.C. § 112(1), the Examiner asserts at page 9 of the Office Action: "*It is not known under what circumstances the result observed in the instantly examined control and test populations would be repeatable, as the results have not been validated.*"

3. As a scientist skilled in the area of molecular biomarker identification, I submit that post-filing validation experiments performed by the Assignee of the present application using both quantitative RT-PCR (QRT-PCR), an alternate technology relative to microarray analysis employed in the experiments disclosed at Example 21 of the specification, as well as an independent cohort of control and disease subjects relative to those employed in the experiments disclosed at Example 21 of the specification, have shown that RNA encoded by the gene ABCA1 is present at statistically lower levels in blood of subjects having bladder cancer relative to healthy control subjects.

Levels of ABCA1-encoded RNA are statistically lower in blood of coronary artery disease patients versus healthy control subjects – validation of ABCA1 as CAD biomarker in blood via quantitative RT-PCR using an alternative cohort

Attached as Exhibit "A" to this Declaration is a disclosure and analysis of post-filing experiments performed by the Assignee in which levels of ABCA1-encoded RNA in blood samples from patients with coronary artery disease (CAD) were found to be statistically lower relative to healthy control subjects, as determined via quantitative RT-PCR (QRT-PCR) using an alternative cohort relative to that employed to generate the results disclosed in the specification.

Quantitative RT-PCR experiments were performed as described under Materials and Methods in Exhibit "A", below, in order to validate results obtained using microarray hybridization analysis described in the specification (Example 21) disclosing differential expression of ABCA1-encoded RNA between blood samples from 19 CAD patients and 14 healthy control subjects. As shown in Tables 1 and 2 and Figure 1 of Exhibit "A", the average level of ABCA1-encoded RNA in samples from 19 CAD patients, as determined via QRT-PCR, was found to be 0.65 that of, i.e. 1.54-fold lower than that of, 14 healthy control subjects tested, with the difference in expression levels being statistically significant ($p = 0.0001$). The signal intensity data for each sample was analyzed via the ROC curve approach to determine the optimal expression level threshold to differentiate between levels of ABCA1-encoded RNA in samples from CAD patients versus healthy control subjects. As can be seen in Table 2 of Exhibit "A", 15 out of 19 CAD samples scored above the classification threshold value, while 10 out of 14 healthy control subject samples scored below the classification threshold value. These results hence signify that ABCA1-encoded RNA levels are statistically lower in blood of CAD patients relative to healthy subjects, such that analysis of such levels can be used to determine that a human test subject is a candidate for having CAD with a specificity of 71% and a sensitivity of 79%.

In view of the above, I submit that the specification enables one of skill in the art to practice the claimed methods.

4. I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that wilful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardize the validity of the application or any patent issuing thereon.

Hongwei Zhang, Ph.D. Zhangwei

Date Nov. 16, 2007

EXHIBIT "A"

TABLE 1. Quantitative RT-PCR analysis of ABCA1-encoded RNA levels in blood of CAD patients vs healthy control subjects. The relative fold-change threshold used for sample classification was: 8.6. Data were obtained as described under Materials and Methods, below. Median fluorescence of controls normalized to a value of "1".

Experimental group	**Level of ABCA1-encoded RNA** (ΔCt)	**Sample classification** (ΔCt ≤ 8.6: "non-CAD"; ΔCt > 8.6: "CAD")
CAD patients	4.585	"non-CAD"
	7.84	"non-CAD"
	8.095	"non-CAD"
	8.235	"non-CAD"
	8.745	"CAD"
	8.805	"CAD"
	9.18	"CAD"
	9.825	"CAD"
	10.005	"CAD"
	10.335	"CAD"
	10.49	"CAD"
	10.585	"CAD"
	10.755	"CAD"
	10.93	"CAD"
	10.945	"CAD"
	10.98	"CAD"
	11.065	"CAD"
	11.575	"CAD"
	12.55	"CAD"
Healthy control subjects	5.7	"non-CAD"
	5.845	"non-CAD"
	7.455	"non-CAD"
	7.665	"non-CAD"
	7.73	"non-CAD"
	7.93	"non-CAD"
	8.075	"non-CAD"
	8.115	"non-CAD"
	8.38	"non-CAD"
	8.535	"non-CAD"
	8.61	"CAD"
	8.88	"CAD"
	9.445	"CAD"
	11.41	"CAD"

TABLE 2. Analysis of QRT-PCR data of Table 1, above, for CAD detection. Statistical analysis was performed as described under Materials and Methods, below.

Fraction CAD samples above threshold ΔCt	15/19
Fraction control samples below threshold ΔCt	10/14
Accuracy	25 / 33 x 100 % = 76 %
Specificity	10 / 14 x 100 % = 71 %
Sensitivity	15 / 19 x 100 % = 79 %
Average fold-change (CAD/control)	0.651 (1/0.651 = 1.54-fold decrease)
***p*-value**	0.0001

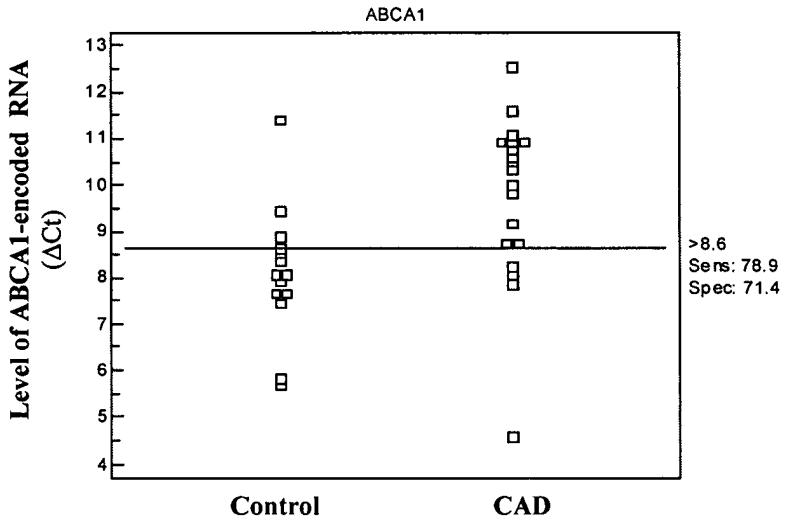

FIG. 1. Levels of ABCA1-encoded RNA in blood samples from CAD patients are on average 1.54-fold lower (p=0.0001) than those of healthy control individuals, as determined via real-time quantitative RT-PCR. The horizontal line represents the classification threshold ΔCt value of 8.6, determined via ROC curve analysis. Data were obtained as described under Materials and Methods, below, and correspond to data shown in Table 1 above.

Materials and Methods:

Blood RNA Isolation: Samples were obtained from 19 patients diagnosed with single or multi-vessel CAD prior to angioplasty, and from 14 healthy control subjects. All participants provided written informed consent. Approximately 10ml of blood was collected from each participant, using a Vacutainer™ tube (Becton Dickinson, Franklin Lakes, NJ). Red blood cells were ruptured with hypotonic lysis buffer (1.6 mM EDTA, 10 mM KHCO3, 153 mM NH4Cl, pH 7.4), followed by collection of white blood cells by centrifugation. White blood cell total RNA was extracted with Trizol® Reagent

(Invitrogen, Carlsbad, CA), according to the manufacturer's instruction. The quality of RNA samples was assessed on an Agilent Bioanalyzer 2100 using RNA 6000 Nano Chips (Agilent Technologies, Palo Alto, CA), and the quantities of RNA were measured by UV spectrophotometry (Beckman-Coulter DU640).

Real-time QRT-PCR: Real-time QRT-PCR was used to measure levels of ABCA1-encoded RNA in blood samples from CAD patients and healthy control subjects. First strand cDNA was synthesized from 1μg total RNA using the ABI High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) in a volume of 100μl, consisting of 10μl 10x RT buffer, 4μl 100mM dNTP mix, 10μl 10x RT random primers and 5μl Multi-scribe reverse transcriptase (50U/μl). Real-time PCR was performed on an ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). PCR was performed in a reaction volume of 25μl consisting of 12.5μl 2X SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 5μl of 5pmol primer mix (forward primer, AGCCCACCAGTACTGTCTCAG; reverse primer, ACTGAGGATTTGCTTCGGTGGT; both primers are optimized for an annealing temperature of 56°C), and 2.5ng first strand cDNA. The PCR cycling protocol used is as follows: (1) 50°C, 2 min; (2) 95°C, 10 min; (3) 40 cycles of 95°C, 15 sec; 60°C, 1 min; and (4) determining the dissociation curve from 60°C to 95°C. The beta-actin gene or 18S rRNA was used as the housekeeping gene for normalization.

Determination of classification thresholds and evaluation of sensitivity and specificity: Receiver operating characteristic (ROC) curve analysis [Pepe MS. The Statistical Evaluation of Medical Tests for Classification and Prediction. Oxford: Oxford University Press; 2003; Metz CE. Basic principles of ROC analysis. Semin Nucl Med 1978; 8: 283-98; Swets JA. Measuring the accuracy of diagnostic systems. Science 1988; 240: 1285-93; Zweig MH, Campbell G. Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. Clin Chem 1993; 39: 561-77] was used to analyze the signal intensity data for each gene to determine the optimal expression level classification threshold for classifying levels of ABCA1-encoded RNA in the samples as being characteristic either of CAD patients or of healthy control subjects. Receiver operating characteristic curve analysis was used to evaluate the TPF (true positive fraction;

sensitivity) and FPF (false positive fraction; 1-specificity). Receiver operating characteristic curves were generated using MEDCALC software.

Statistical analysis: A Mann-Whitney rank sum test was used, using the fold-change values, to test for the statistical significance of the difference in RNA levels between the disease and healthy control groups. Fold change was calculated using the following formula: $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct$ was calculated by subtracting the mean ΔCt value of the control samples from the ΔCt of each sample for each gene. Statistical analysis was performed using SigmaStat v3.0 (SPSS Scientific, Chicago, IL).